

Heterogeneity of *erbB-2* Gene Amplification in Bladder Cancer¹

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Abstract

erbB-2 amplification and overexpression have been suggested as potentially useful prognostic markers in bladder cancer. We examined 141 bladder tumor specimens (45 fresh tissue samples and 96 formalin fixed tissue blocks) for *erbB-2* amplification using fluorescence *in situ* hybridization. A dual labeling hybridization using a repetitive pericentromeric probe specific for chromosome 17 and a cosmid probe for the *erbB-2* locus was performed to analyze the *erbB-2* copy number in relation to chromosome 17 copy number on a cell by cell basis. Amplification (more than twice as many *erbB-2* signals as centromere 17 signals per tumor) was found in 10 of 141 tumors. There was considerable heterogeneity in *erbB-2* amplification. In a given tumor there was a wide range of *erbB-2* copy number in amplified cells. The arrangement of *erbB-2* signals in clusters in all amplified cases suggests that *erbB-2* amplification occurs intrachromosomally in bladder cancer. Amplification was found only in tumors with aneusomy of chromosome 17 and was more frequent in pT2-T4 tumors than in pTa/T1 tumors. Overexpression was present without amplification in 51 tumors. All tumors with *erbB-2* amplification showed *erbB-2* overexpression. However, in 5 samples the proportion of cells with amplification was significantly lower than the fraction of cells with overexpression, indicating coexistence of two different mechanisms leading to overexpression in these tumors.

Introduction

The *erbB-2* gene, located on chromosome 17q21 (1), specifies a *M*_{185,000} transmembrane phosphoglycoprotein that is closely related to the epidermal growth factor receptor. Overexpression of the *erbB-2* protein is a frequent and prognostically relevant event in a variety of human tumors (2-7). Although DNA amplification is the predominant mechanism of *erbB-2* protein overexpression in breast cancer (8, 9) this pathway seems to be less frequent in cancer of the bladder (10, 11) and esophagus (6). Studies of *erbB-2* overexpression and amplification have shown varying results in bladder cancer (10-15). The proportion of cases reported showing *erbB-2* overexpression has ranged from 2 to 70% (10-12, 14-16).

Kallioniemi *et al.* (8) recently described the detection of *erbB-2* amplification in breast cancer by FISH.³ FISH allows the detection of gene amplification on a cell by cell basis and is therefore especially suited to examine gene amplification in tissue samples harboring heterogeneity of amplification. Tumor heterogeneity is thought to reflect genetic instability and may be an important characteristic of solid tumors since genetic instability is essential for tumor evolution.

In this study we used FISH to clarify the incidence of *erbB-2* amplification and to determine to what extent there is heterogeneity of *erbB-2* amplification and overexpression in bladder cancer.

Materials and Methods

Patient Material. Both fresh imprint preparations and cells dissociated from formalin fixed paraffin embedded thick sections (50 μ m) were prepared for analysis as described previously (17, 18). Tumor blocks chosen for analysis contained at least 60% tumor cells in all cases. Tumor stage and grade were defined according to International Union against Cancer (19) and WHO (20) classifications. Because of the limitations of transurethral biopsies in accurately determining the depth of invasion of higher stage bladder cancer all tumors showing muscle invasion were categorized into one group (pT2-T4).

Immunocytochemistry. Formalin fixed tissue sections were stained for *erbB-2* expression using a polyclonal antibody (DAKO, Glostrup, Denmark; 1:1000) and standard indirect immunoperoxidase procedures. Breast tumors with known overexpression of *erbB-2* were used as positive controls. Positive *erbB-2* staining was defined as at least 10% of tumor cells with distinct membranous staining to exclude false positivity due to crush or edge artifact. Tumors with *erbB-2* amplification detected by FISH were further reviewed and the percentage of all tumor cells staining for *erbB-2* on the entire slide was estimated in intervals of 10%. BrdUrd incorporation was measured as the BrdUrd LI as described previously (17).

DNA Probes for FISH. A probe specific for the chromosome 17 pericentromeric sequence (p17H8) was used in combination with two contiguous cosmid probes specific for *erbB-2*. A cosmid probe for the *p53* locus on 17p was also used for selected cases. Cosmid DNA was labeled with digoxigenin-11-dUTP and centromeric DNA was labeled with biotin-14-dATP by nick translation using standard protocols.

Fluorescence *In Situ* Hybridization. All slides were fixed in methanol:acetic acid (3:1) and subsequently air dried. FISH was performed as described previously (8, 18) with modifications. Cells on slides were denatured in 70% formamide in 2 \times SSC, pH 7, at 75°C for 2.5 min. After dehydration in graded ethanol, samples were treated with proteinase K (Sigma). Touch preparations were treated at 0.5 μ g/ml, while dissociated cells received 2.0 μ g/ml in phosphate buffered saline (pH 7.0) for 7 min at 37°C, followed by ethanol dehydration. The hybridization mixture was denatured for 5 min at 75°C, allowed to reanneal for 30 min at 37°C, and applied to denatured cells on slides. Ten μ l of hybridization mixture consisted of 10 ng of cosmid probe, 4 ng of centromeric probe (20 ng for formalin fixed cells) as well as 10 ng of unlabeled, sonicated (200-500 base pairs) human placental DNA (Sigma) in 50% formamide, 10% dextran sulfate, and 2 \times SSC (pH 7). Hybridization was overnight at 37°C. Visualization of hybridization signals was as described (8, 18). Lymphocyte controls were used to assure probe specificity. If tumor cell signals were weak, presumably due to low hybridization efficiency, hybridization was repeated using the same protocol with an increased proteinase K concentration (up to 15 μ g/ml). Proteinase K concentration was reduced if excessive nuclear damage was observed because *erbB-2* signals were sometimes split in damaged cells.

Scoring of FISH Signals. Cells were selected for scoring according to morphological criteria using 4,6-diamidino-2-phenylindole staining. For imprint preparations, cells in groups or sheets were selected because these were considered to be epithelial in origin. For dissociated cells, only those cells having a malignant cytological appearance (especially large cells) were scored. Copy numbers of both centromere 17 and *erbB-2* signals were counted for 50-100 nuclei. Since the optimum protease K concentration varied greatly between cases the use of a reliable external control was not possible. Therefore, to avoid misinterpretation due to insufficient hybridization efficiency, cells were scored only when at least one bright cosmid and one bright centromere signal were present. A tumor was considered amplified if the average *erbB-2* copy number per centromere 17 per scored cell exceeded 2. An individual

Received 1/28/93; accepted 4/5/93.

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¹ Supported by NIH Grant CA47537, Schweizerischer Nationalfonds, Janggen-Poehn-Stiftung, Holderbank-Stiftung, Schweizerische Krebsliga, and Ciba-Geigy Jubilaeumsstiftung (G. S.).

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³ The abbreviations used are: FISH, fluorescence *in situ* hybridization; BrdUrd, 5-bromo-deoxyuridine; LI, labeling index; SSC, standard saline-citrate (1 \times SSC is 0.15 M NaCl-0.015 M sodium citrate).

tumor cell was counted as amplified if there were more than twice as many *erbB-2* signals as centromere 17 signals.

Statistics. A test for linear trend in proportions was applied to examine the relationship of *erbB-2* amplification and aneusomy 17 with tumor stage and grade. A χ^2 test was performed to examine the relationship of *erbB-2* amplification with protein expression, elevated *erbB-2* gene copy number and aneusomy. A Mann-Whitney *U* test was used to compare BrdUrd LI with aneusomy 17. The relationship between average *erbB-2* copy number and BrdUrd LI was evaluated with a Spearman rank correlation test.

Results

Pathology. One hundred forty-one bladder tumors (96 paraffin dissociated and 45 fresh touch preparations) were successfully analyzed by FISH (10 additional dissociated cases and 9 fresh cases had insufficient hybridization intensity and were excluded). There were samples from 131 patients consisting of 127 primary tumors and 14 recurrences. Twenty-six were confined to the bladder mucosa (pTa), 42 showed invasion of the lamina propria (pT1), and 67 were muscle invasive (pT2-T4). Twenty tumors were classified as grade 1, 39 were grade 2, and 46 were grade 3. Grading and staging could not unequivocally be performed in another 6 cases because of inadequate biopsy size or mechanical damage. BrdUrd LI was analyzed in 32 cases and ranged from 2 to 30% (mean, 16.6%). As reported previously for a larger series of tumors which included the present set there was a significant trend for higher BrdUrd LI in higher stage and higher grade tumors (17).

erbB-2 Amplification. In most of the examined tumors the *erbB-2* gene copy number was closely associated with the chromosome 17 copy number on a cell by cell basis. Most of the scored tumor cells contained equal numbers of centromere 17 and *erbB-2* signals. However, there was always a small fraction of cells (<30%) with more *erbB-2* signals than centromere 17 signals. In these cells, extra *erbB-2* signals were usually due to closely associated "paired" signals, presumably representing sister chromatids in S or G₂.

Ten cases showed cells with high levels of *erbB-2* copy number. Considerable heterogeneity in *erbB-2* copy number per cell was seen within each of these cases. *erbB-2* copy number reached more than 20 copies in all 10 tumors and more than 50 copies in 7 tumors (Fig. 1). In all of these cases distinct clusters of *erbB-2* signals (i.e., a group of 7 or more signals lying close together) were seen (Fig. 2A). All 10 tumors fulfilled our criteria for amplification (i.e., the average *erbB-2*

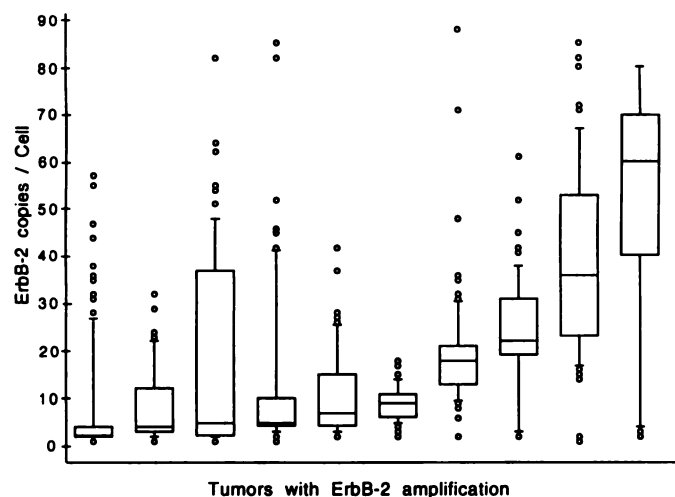


Fig. 1. *erbB-2* copy number in amplified bladder tumors. Values noted for each distribution are 10th percentile (lower small bar), 25th percentile (bottom of box), 50th percentile (center bar), 75th percentile (top of box), and 90th percentile (upper small bar). Events below the 10th and above the 90th percentile are plotted. Fifty–100 cells scored per tumor.

copy number per centromere 17 per tumor cell was greater than 2). To define the fraction of amplified cells in these 10 tumors the proportion of cells showing more than twice as many *erbB-2* signals as centromere 17 signals was determined. This fraction of amplified cells ranged from 18 to 94% in these tumors (Table 1). Intratumor heterogeneity is further evident in these cases by the presence of polysomic (i.e., neoplastic) cells which were not amplified (0–63% in these tumors).

Twelve cases showed a borderline result with an *erbB-2* copy number per centromere 17 per tumor cell between 1.5 and 2.0. No *erbB-2* clusters were present in these tumors. Included in this group were 2 cases in which the predominant population of cells had 2 centromeres 17 and 3 *erbB-2* signals (Fig. 2B). Using a probe for 17p instead of *erbB-2* the predominant population showed 2 centromeres and 1 cosmid in both cases, suggesting the presence of isochromosome 17q.

erbB-2 Protein Expression. Fifty of 59 (85%) tumors with *erbB-2* overexpression showed no *erbB-2* amplification. Fig. 3A illustrates that normal (i.e., 2 *erbB-2* copies/cell) or only slightly elevated total *erbB-2* copy number was the predominant finding in this series of bladder tumors. Using a cutoff level of 2.5 for *erbB-2* gene copy number per cell, an increased *erbB-2* copy number was found in 37 of 52 (71%) *erbB-2* protein positive tumors but in only 14 of 34 (41%) *erbB-2* protein negative tumors ($P = 0.0057$, χ^2 test), when the cases with amplification were excluded. Aneusomy (average centromere 17 per cell >2.3) was also more frequent among tumors with *erbB-2* overexpression (43 of 60) as compared to tumors without detectable *erbB-2* expression (17 of 35; $P = 0.0013$, χ^2 test; cases with amplification excluded). The relationship between *erbB-2* copy number per centromere 17 per cell and *erbB-2* protein expression is shown in Fig. 3B. Most tumors showed a consistently low *erbB-2* copy number per centromere 17, independent of the *erbB-2* expression level. Of five cases with *erbB-2* copy number per centromere 17 between 1.5 and 2.0 three showed protein overexpression and two did not.

All 9 amplified cases (*erbB-2* copy number per centromere 17 greater than 2) had *erbB-2* overexpression. However, there was a dissociation seen between *erbB-2* amplification and the level of protein overexpression (Table 1). The percentage of cells with overexpression and amplification was similar in 4 of 9 amplified tumors. In the other 5 samples the proportion of cells with amplification was distinctly lower than the proportion of cells showing *erbB-2* overexpression (Figs. 2, C and D). Cells without *erbB-2* amplification still showed centromere 17 aneusomy, evidence of their neoplastic nature. In the patient where primary tumor (case 7) and recurrence (case 8) were examined, the proportion of amplified cells was higher in the recurrence, which occurred 5 months after the primary tumor.

erbB-2 Amplification versus Grade and Stage. *erbB-2* amplification was found in 0 of 26 pTa, 1 of 42 pT1, and 9 of 64 pT2-T4 tumors ($P = 0.013$) as well as in 1 of 28 grade 1, 3 of 51 grade 2, and 6 of 56 grade 3 tumors ($P = 0.179$). The average centromere 17 copy number and the fraction of cells with abnormal centromere 17 copy number are reflective of chromosome 17 aneusomy. Both parameters showed a strong correlation to tumor grade and stage ($P = 0.0001$, Kruskal-Wallis analysis). Aneusomy was found in 3 of 26 pTa tumors, 18 of 42 pT1 tumors, and 49 of 67 pT2-T4 tumors ($P < 0.0001$) as well as in 5 of 23 grade 1, 29 of 51 grade 2, and 43 of 57 grade 3 tumors using a cutoff level of 2.3 for average centromere 17 copy number ($P < 0.0001$). Similar results were obtained when aneusomy was defined as fewer than 75% cells with 2 centromeres 17/cell. All 10 tumors with *erbB-2* amplification showed aneusomy for chromosome 17.

BrdUrd Labeling. BrdUrd labeling was done in only a subset of tumors (32 of 141). BrdUrd LI was higher in tumors with aneusomy

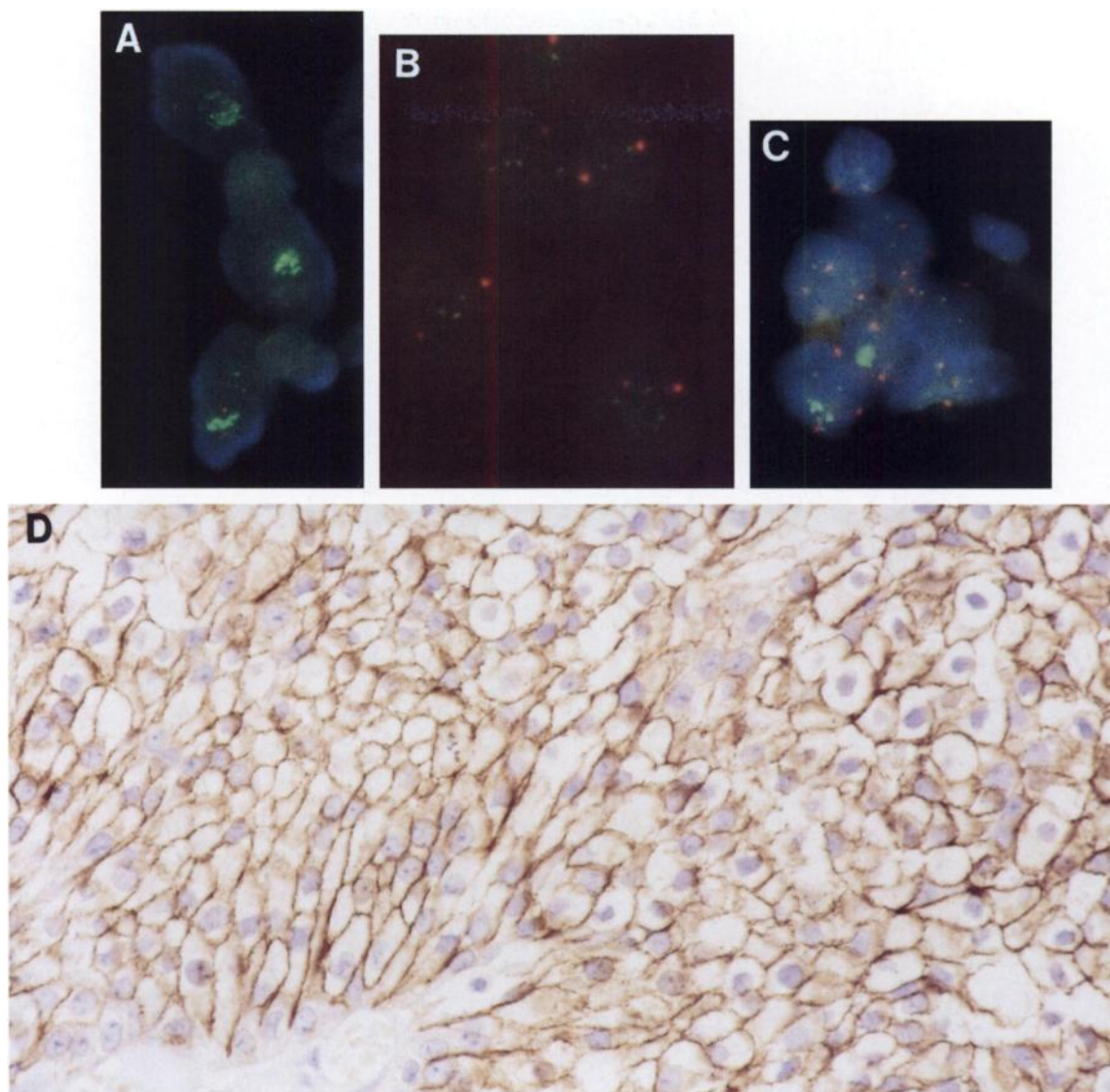


Fig. 2. *erbB-2* FISH and immunostaining. A–C, *in situ* hybridization with *erbB-2* cosmid contig. 4,6-Diamidino-2-phenylindole counterstaining (blue), *erbB-2* signal (green), centromere 17 (red). A, clusters of *erbB-2* signals in 3 amplified cells (note smaller cells without amplification). B, pattern suggestive of isochromosome 17q, showing 2 centromeres 17 and 3 *erbB-2* signals. C, heterogeneous pattern of *erbB-2* amplification (clusters) in 2 cells of a group. Note cells with 3 centromeres 17 and no amplification. D, *erbB-2* immunostaining of the same case as in C. Virtually every cell is immunopositive.

17 than in disomic tumors (18.8 ± 6.9 versus 7.0 ± 6.7 ; $P = 0.0047$, *U* test) when aneusomy was defined as 2 centromeres 17 in less than 90% of scored cells. Only one of the tumors labeled with BrdUrd showed *erbB-2* amplification. The BrdUrd LI was especially high in this case (30.0%), compared to a mean LI of 16.6%. There was no correlation between average *erbB-2* copy number and BrdUrd LI (Spearman rank correlation).

Discussion

These data show that *erbB-2* amplification is not a frequent cause of *erbB-2* overexpression in bladder cancer. Moreover, in several samples where amplification was present there was a dissociation between gene copy number and *erbB-2* overexpression. The fraction of cells with amplification in these cases was distinctly lower than the fraction of cells with overexpression, suggesting that multiple mechanisms leading to overexpression may coexist within a single tumor.

An average *erbB-2* copy number per centromere 17 scored cell greater than 2 was selected to define tumor amplification in this study. This cutoff was established based on previous studies in breast cancer

and by analogy to other molecular techniques. Our group has validated the technique in a previous study showing a strong correlation between amplification analysis by FISH (using this definition for amplification) and slot blot analysis (8). In that study, our definition identified all cases in which at least a subpopulation of cells showed an obviously increased *erbB-2* copy number. Considering the striking heterogeneity of *erbB-2* copy number in tumor cells and the confounding effect of contaminating normal cells, average *erbB-2* copy number may be suboptimal as a measure of gene amplification. FISH allows additional characterization of amplification in a given tumor by providing measures of the fraction of amplified cells and the level of amplification within the amplified cells. The fraction of amplified cells ranged from 18 to 94% in this study. The biological significance of smaller fractions of tumor cells with amplification is not known and might be investigated in further FISH studies.

In this study distinct clusters of *erbB-2* signals were found in cells of all tumors with amplification. Clusters of *erbB-2* signals have also been found by Kallioniemi *et al.* (8) in all cases with *erbB-2* amplification. They may most likely represent homogeneously staining

Table 1 Tumors with erbB-2 amplification

Case	Stage ^a	Grade	erb/c17 ^b	Av. c17 ^c	erbB-2 IHC ^d (%)	Amplified cells ^e (%)	Cells with polysomy 17 ^f (%)		Cells with mono-/disomy 17 ^g (%)	
							Amplified ^e	Not amplified	Amplified ^e	Not amplified
1	2	2	2.30	4.08	40	49	43	47	6	4
2	2	3	3.63	11.16	100	80	80	16	0	4
3	2	3	6.47	3.73	100	88	82	4	6	8
4	2	3	6.43	2.99	100	94	76	0	18	6
5	2	2	2.80	3.09	80	29	20	43	9	28
6	1	1	2.23	2.58	100	18	16	16	2	66
7	2	3	3.49	3.57	100	28	25	63	3	9
8 ^h	2	3	4.13	3.59	90	41	41	27	0	32
9	2	2	2.51	4.40	100	38	37	53	1	9
10	2	3	19.06	2.75	nd ⁱ	89	68	10	21	1

^a Pathological stage.
^b Average erbB-2 signal/centromere 17 signal/cell.
^c Average centromere 17 copy number/cell.
^d Percentage of cells staining positively for erbB-2 by immunohistochemistry.
^e Percentage of cells with at least 2 times the number of erbB-2 as centromere 17 signals.
^f Cells with 3 or more centromere 17 signals.
^g Cells with 1 or 2 centromere 17 signals.
^h Recurrence from case 7 (after 5 months).
ⁱ ND, not done; c17, centromere 17; IHC, immunohistochemistry.

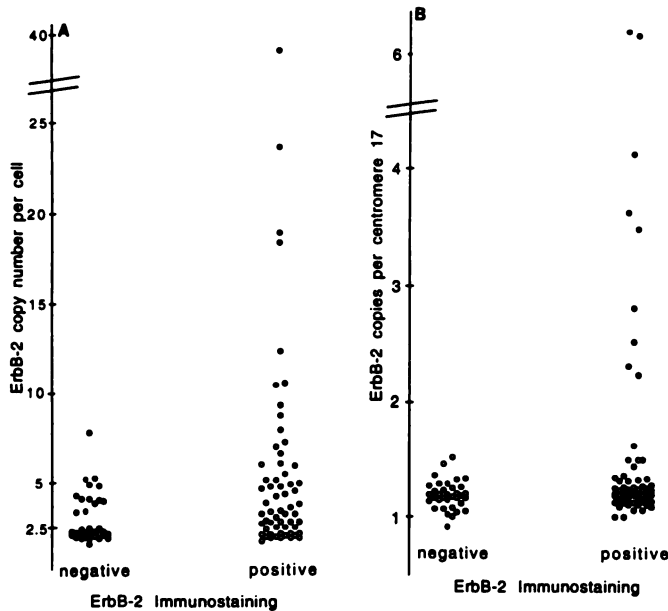


Fig. 3. erbB-2 gene copy number versus protein overexpression. ○, tumors without amplification; ●, cases with amplification (more than twice as many erbB-2 signals as centromere 17 signals). A, total erbB-2 copy number/cell; B, erbB-2 copy number/centromere 17/cell.

regions. Our data suggest that homogeneously staining regions are the major representations of erbB-2 amplification in bladder cancer, as in breast cancer.

The prevalence of amplification in this study is similar to the results of Zhau *et al.* (11), who found amplification in 2 of 24 high grade bladder cancers that was slightly lower than reported by Coombs *et al.* (10), who showed amplification in 6 of 13 high grade tumors. However, Coombs *et al.* (10) used a probe for the (frequently deleted) p53 gene as one of their reference probes, perhaps leading to an overestimate of erbB-2 amplification. The presence of isochromosome 17 or a physical deletion of one p53 allele together with a slightly elevated erbB-2 copy number could also result in an erbB-2 amplification of 3-fold or more by Southern blot analysis. In the present study a pattern suggestive of isochromosome 17q was seen, as well as a number of cases with slightly elevated erbB-2 copy number per centromere 17.

Our data show that increased erbB-2 copy number either may be

due to an increased copy number of the entire chromosome 17 or may be independent of chromosome copy number. Whereas all cases with amplification (erbB-2 copy number per centromere 17 greater than 2) showed erbB-2 protein overexpression, the meaning of increased erbB-2 copy number due to chromosome 17 aneusomy is less clear. Although there is a weak correlation between chromosome 17 copy number per cell and erbB-2 protein overexpression, it is not clear that these copy number alterations lead to protein overexpression. The fact that many cases with high total erbB-2 copy number showed no erbB-2 overexpression and many cases with low erbB-2 copy number had erbB-2 overexpression indicates that aneusomy 17 is neither mandatory nor sufficient for erbB-2 overexpression. Because there is a strong association between centromere 17 copy number and copy number of other chromosomes in our tumor material (data not shown) we assume that aneusomy 17 represents DNA aneuploidy rather than being an independent genetic event. Since aneuploidy is a feature of genetic instability it might be associated with another yet unknown genomic alteration eventually leading to erbB-2 overexpression. In agreement with Coombs *et al.* (10) and Zhau *et al.* (11) erbB-2 amplification was seen in only a minor fraction of cases showing overexpression.

It is well known that oncogene overexpression may be due to gene amplification, point mutation, translocation, or transcriptional up-regulation. Assuming that only one mechanism would be active in a single tumor, we expected to find a similar number of amplified cells as overexpressing cells in cases with erbB-2 amplification. Surprisingly, the proportion of amplified cells was markedly lower than the fraction of overexpressing cells in 5 of 9 samples with erbB-2 amplification. This discrepancy was not due to an admixture of nonneoplastic cells, since in all of these cases a significant fraction of non-amplified cells had more than 2 centromere 17 copies. We therefore conclude that in these tumors two populations of erbB-2 overexpressing cells coexist, one with gene amplification and one without amplification. It appears possible that amplification might follow previous overexpression by another cause possibly providing further growth advantage to that tumor subpopulation. This latter model is supported by the observation that in one case a recurrence showed a higher fraction of amplified cells than the primary tumor 5 months earlier. Alternatively amplification may be the primary event and may be associated with transcriptional up-regulation in neighboring cells. Further studies examining the fraction of amplified cells in subsequent biopsies of patients with known erbB-2 amplification will give further insights into the dynamics of development of erbB-2 amplification.

Acknowledgments

Cosmid probes were kindly provided by Drs. Richard M. Cawthon, Peter O'Connel, and Ray White (Howard Hughes Medical Institute, Salt Lake City, UT). The authors thank S. DeVries, R. Abrecht, I. Epper, and the staff of the Pathology Department, University of Basel, for their technical support.

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